Sense transformation reveals a novel role for class I \( \beta \)-1,3-glucanase in tobacco seed germination

Gerhard Leubner-Metzger* and Frederick Meins Jr
Friedrich Miescher-Institute, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

Received 21 March 2000; accepted 5 April 2000.
*For correspondence (fax +41 61 697 3978; e-mail leubner@fmi.ch).

Summary

‘Coat-enhanced’ seed dormancy of many dicotyledonous species, including tobacco, is released during after-ripening. Rupture of the endosperm, which is the limiting step in tobacco seed germination, is preceded by induction of class I \( \beta \)-1,3-glucanase (\( \beta \)GLU1) in the micropylar endosperm where the radicle will penetrate. Treating after-ripened tobacco seeds with abscisic acid (ABA) delays endosperm rupture and inhibits \( \beta \)GLU1 induction. Sense transformation with a chimeric ABA-inducible \( \beta \)GLU1 transgene resulted in over-expression of \( \beta \)GLU1 in seeds and promoted endosperm rupture of mature seeds and of ABA-treated after-ripened seeds. Taken together, these results provide direct evidence that \( \beta \)GLU1 contributes to endosperm rupture. Over-expression of \( \beta \)GLU1 during germination also replaced the effects of after-ripening on endosperm rupture. This suggests that regulation of \( \beta \)GLU1 by ABA signalling pathways might have a key role in after-ripening.

Keywords: abscisic acid, after-ripening, endosperm-limited seed germination, gene function, \( \beta \)-1,3-glucanase, sense transformation.

Introduction

Little is known about the molecular basis for the after-ripening and germination of dicotyledonous seeds (Bewley, 1997a). During seed maturation, water content decreases, abscisic acid (ABA) accumulates, and primary dormancy is established (Hilhorst, 1995; Li and Foley, 1997; Rock and Quatrano, 1995). Primary dormancy of many species can be altered by after-ripening, i.e. the storage of mature seeds for several months under dry, warm conditions (Hilhorst, 1995; Kasperbauer, 1968; Koornneef and Karsse, 1994; Li and Foley, 1997). Under favourable conditions, imbibition of water by non-dormant seeds results in a burst of respiration, rapid growth of the embryo, rupture of the covering layers, and finally emergence of the radicle. Many of these seeds exhibit ‘coat-enhanced’ dormancy in which the emergence of the radicle is physically restrained by the covering layers (Baskin and Baskin, 1998; Bewley, 1997a; Hilhorst, 1995). In the case of endosperm-limited germination, it is believed that hydrolytic enzymes facilitate weakening of the endosperm surrounding the radicle tip by hydrolysing cell-wall materials (Bewley, 1997b; Black, 1996; Ni and Bradford, 1993; Sirit et al., 1999); however, direct evidence for this hypothesis from studies with transgenic seeds is lacking.

The first morphological event following imbibition of tobacco seeds is rupture of the seed coat (testa). This is followed by rupture of the endosperm, which is the limiting step of tobacco seed germination (Arcila and Mohapatra, 1983; Leubner-Metzger et al., 1995). \( \beta \)-1,3-glucanase (\( \beta \)GLU1) activity is induced after testa rupture and just prior to the onset of endosperm rupture. This activity is localized in the micropylar endosperm at the site where the radicle will emerge, and results from transcriptional induction of class I \( \beta \)-1,3-glucanase (\( \beta \)GLU1) (Leubner-Metzger et al., 1995). The induction of \( \beta \)GLU1 and endosperm rupture are tightly linked in response to physiological factors known to affect the incidence and timing of germination (Leubner-Metzger et al., 1996; Leubner-Metzger et al., 1998). For example, ABA treatment specifically delays endosperm rupture and inhibits the induction of \( \beta \)GLU1. Kinetic analysis of this effect strongly suggests that the onset of endosperm rupture depends on a critical threshold concentration of \( \beta \)GLU1.

In the present study, we established by sense transformation that \( \beta \)GLU1 has a causal role in endosperm rupture. We also provide less direct evidence that after-ripening
could affect endosperm rupture, at least in part, by a βGLU I-mediated process.

Results

High-level expression of βGLU I can promote endosperm rupture

Tobacco plants were transformed with the expression vector pKS7 carrying a chimeric tobacco βGLU I gene regulated by the castor bean Cat1 gene promoter. The Cat1 promoter was chosen because it directs high-level expression of a reporter gene in the endosperm of germinating seeds of transgenic tobacco (Suzuki et al., 1995). Unless indicated otherwise, after-ripened seeds obtained by self-fertilization of primary sense (TKS7) and empty-vector (TCB1) transformants were used. Seeds were imbibed in the presence of ABA to delay the onset of germination and inhibit the induction of host βGLU I genes (Leubner-Metzger et al., 1995).

In our initial screen, we scored the incidence of germination, i.e. percentage of endosperm rupture, at 73h after the start of imbibition in the presence of 1μM ABA in continuous light. Under these conditions, the germination rate of the 27 empty-vector TCB1 lines used as controls was 6.8 ± 1.2% (mean ± SE) and the population of seeds contained a mean βGLU I activity of 0.9 ± 0.1 pkat/seed. In contrast, 41.9% of the 43 TKS7 lines germinated at rates at least twofold higher than the TCB1 controls and exhibited a 7.5-fold higher mean βGLU I activity. Thus, germination was correlated with elevated βGLU I activity in numerous, independent sense transformants.

Four representative TKS7 lines with high germination rates in the presence of 1μM ABA were chosen that differed in transgene dose and βGLU I content. The time course for testa rupture, endosperm rupture and βGLU I activity accumulation was determined for lines carrying one (TKS7-43), two (TKS7-34 and TKS7-42) and three or more (TKS7-10) transgene loci and for four control TCB1 lines imbibed with and without 10μM ABA in the incubation medium. The time course for testa rupture was not affected by ABA treatment and was very similar for TKS7 and TCB1 seeds (data not shown). Figure 1(a) shows that after-ripened seed of TKS7 and TCB1 lines

![Figure 1](https://via.placeholder.com/150)

did not differ in timing of endosperm rupture when imibed in medium without added ABA. As reported earlier for wild-type seeds (Leubner-Metzger et al., 1995), treatment with 10μM ABA dramatically delayed by approximately 70h the time for 50% endosperm rupture of TCIB1 seeds (Figure 1c). This delay in endosperm rupture was substantially reduced by about 15h in TKSG7 seeds.

Three of the TKSG7 lines accumulated considerably more βGLU activity than did the empty-vector control lines when imibed without added ABA. The activity of line TKSG7-43 was comparable to the TCIB1 lines (Figure 1b). The finding that over-expression of βGLU I in sense lines did not promote endosperm rupture under these conditions is consistent with studies showing that βGLU activities above approximately 5 pkat/seed at the onset of rupture do not increase the incidence of endosperm rupture (Leubner-Metzger et al., 1995). ABA treatment inhibited βGLU accumulation of TCIB1 seeds (Figure 1d) as reported earlier for wild-type seed (Leubner-Metzger et al., 1995). In contrast, ABA treatment markedly increased the βGLU activities of the TKSG7 seeds. Although the level of βGLU activity and the induction in response to 10μM ABA differed considerably for the different TKSG7 lines, even the least responsive line, TKSG7-43, showed at least fivefold higher βGLU activities relative to the TCIB1 controls throughout the experiment. Quantitative immunoblot analysis using antibodies that detect all known βGLU classes and RNA-blot hybridization using a probe specific for βGLU I mRNA confirmed that the increased βGLU activity of the TKSG7 lines is due exclusively to increased amounts of βGLU I (data not shown). Taken together, these findings indicate that expression of βGLU I under the regulation of the Cat I promoter increases βGLU I levels during endosperm rupture and results in a partial reversal of the delay in rupture due to ABA.

After-ripening and photodormancy effects

We compared germination in the light of ‘fresh’ seeds, i.e. mature seeds 40 days after pollination (DAP), and of after-ripened seeds stored at room temperature for at least 6 months after harvest. When imibed without added ABA, essentially all tobacco seeds eventually germinate. Thus, changes in the onset of germination can be detected by measuring the germination rate at a fixed time after the start of imbibition. Table 1 shows that after-ripening increased the germination rate of wild-type and TCIB1-2 seed. This indicates that after-ripening can release dormancy of Havana 425 tobacco seeds. Fresh sense seeds (TKSG7) germinated at approximately 1.5-fold higher rates than did the controls, whereas after-ripened sense and control seeds germinated at comparable rates. The βGLU activity at the time of endosperm rupture was higher in after-ripened than in fresh control seeds. Independent of after-ripening, the βGLU content of sense-transformed seeds was higher than that of the control seeds. Taken together, these results suggest that over-expression of βGLU can replace the effect of after-ripening on endosperm rupture in the light.

Havana 425 tobacco seeds exhibit photodormancy, i.e. fresh seeds do not germinate in the dark, even after prolonged periods of time (Leubner-Metzger et al., 1996). Table 1 shows that after-ripening contributes to the release of photodormancy and that this effect varies greatly for different seed batches as reported for other tobacco cultivars (Kasperbauer, 1986). Sense transformation did not have detectable effects on either photodormancy of

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**Table 1. Effect of after-ripening on endosperm rupture, βGLU activity and photodormancy of sense βGLU I seeds**

<table>
<thead>
<tr>
<th>Lines</th>
<th>Continuous light</th>
<th></th>
<th>Darkness</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endosperm rupture (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>βGLU (pkat/seed)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>Non-photodormancy (%)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>‘Fresh’ seed</td>
<td>After-ripened seed</td>
<td>‘Fresh’ seed</td>
<td>After-ripened seed</td>
<td>‘Fresh’ seed</td>
</tr>
<tr>
<td>Wild-type</td>
<td>49.8 ± 5.2</td>
<td>79.2 ± 6.8</td>
<td>2.4 ± 0.1</td>
<td>3.2 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>TCIB1-2</td>
<td>49.7 ± 2.1</td>
<td>81.4 ± 5.2</td>
<td>2.2 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>TKSG7-32</td>
<td>74.4 ± 2.1</td>
<td>79.0 ± 2.4</td>
<td>9.1 ± 0.2</td>
<td>5.3 ± 0.2</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>TKSG7-38</td>
<td>77.1 ± 2.1</td>
<td>73.5 ± 0.1</td>
<td>8.9 ± 0.2</td>
<td>4.9 ± 0.3</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>TKSG7-43</td>
<td>75.3 ± 3.6</td>
<td>75.8 ± 7.4</td>
<td>8.2 ± 0.5</td>
<td>4.7 ± 0.4</td>
<td>0.4 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Wild type and independent, homozygous, monogenic vector-control (TCIB1) and sense βGLU I (TKSG7) tobacco lines selected from the <i>S</i> <i>S</i> seed generation. <sup>b</sup>Mean ± SE of ‘fresh’ (directly after harvest) and after-ripened (>6 months of dry storage) seed from 3-6 capsules scored 72h and 67h, respectively, after the start of imbibition in continuous light in control medium without antibiotics. <sup>c</sup>Mean ± SE βGLU activities of protein extracts prepared from seed samples described under <sup>b</sup>, <sup>d</sup>Mean ± SE of ‘fresh’ and after-ripened seed from 3-6 capsules scored after 10 days incubation in the dark in control medium without antibiotics. The range obtained with different capsules is shown in parentheses.

fresh seed or on the release of photodormancy due to after-ripening.

**Discussion**

**βGLU1 contributes to endosperm rupture**

βGLU1 induction in the micropylar endosperm is tightly correlated with endosperm rupture under a variety of physiological conditions that delay or promote tobacco-seed germination (reviewed in Leubner-Metzger and Meins, 1999). In the present study, we provide direct evidence that βGLU1 has a role in endosperm rupture. The castor bean *Cat1* promoter shown earlier to confer micropylar-endosperm expression in tobacco seeds (Suzuki *et al.*, 1995) was used to regulate expression of a βGLU1 sense transgene. Expression of maize and *N. plumbaginifolia* orthologues of the castor bean *Cat1* gene is strongly induced by ABA during seed maturation and germination (Bueno *et al.*, 1998; Guan and Scandalios, 1993; Willekens *et al.*, 1994; Williamson and Scandalios, 1992). We found that ABA treatment induces the castor bean *Cat1* promoter in tobacco seeds. This effect was exploited to achieve high-level βGLU1 expression in seeds treated with ABA to delay endosperm rupture and block host βGLU1 expression. The results indicate that expression of βGLU1 reduced the delay in endosperm rupture of after-ripened TKSG7 seeds due to ABA treatment, but did not affect the onset of testa rupture. Increased βGLU1 expression also promoted germination of fresh TKSG7 seeds that were not treated with ABA. Taken together, these results show that βGLU1 substantially contributes to endosperm rupture, which is the limiting step in tobacco seed germination.

We also attempted to block βGLU1 induction during endosperm rupture by antisense transformation using the *Cat1* promoter. As is often the case for antisense experiments, this approach was not successful. Although numerous independent transformants were screened, none were found with reduced βGLU activity, βGLU1...
protein or βGLU1 mRNA levels, and none showed effects on endosperm rupture.

In cases of endosperm-limited germination, radicle emergence depends on both wall weakening and sufficient growth of the embryo to overcome the mechanical resistance of the endosperm (Bewley, 1997b; Ni and Bradford, 1993). Ultrastructural studies suggest that the endospermic hole formed at the micropylar end of tobacco seeds results from endosperm ‘dissolution’ rather than from the ‘pushing’ action of radicle growth (Arcila and Mohapatra, 1983). Earlier studies showed that ABA treatment of wild-type tobacco seeds delayed the onset of endosperm rupture and inhibited βGLU1 induction in a dose-dependent fashion (Leubner-Metzger et al., 1995). Independent of the ABA concentration, the onset of endosperm rupture was correlated with a βGLU activity of roughly 2 pkat/seed, and the incidence of endosperm rupture did not increase with βGLU activity greater than roughly 5 pkat/seed. In the present experiment, the onset of endosperm rupture of control TCI1 seeds treated with 10 μM ABA was somewhat lower, roughly 1 pkat/seed. The important point is that all independent sense lines exhibited earlier germination and βGLU activities above this threshold value. This further supports the hypothesis that a threshold βGLU content is required for endosperm rupture, and suggests that accumulation of βGLU is necessary but not sufficient for endosperm weakening.

The mechanism underlying the effect of βGLU1 on endosperm weakening is not known. One possibility is that βGLU1 helps to hydrolyse extracellular polysaccharides important for strengthening the endosperm wall. This would imply that βGLU1, which is a vacuolar protein (Keefe et al., 1990), can be alternatively targeted for secretion. Recent studies suggesting that tobacco βGLU1 (Kunze et al., 1998) and other vacuolar proteins (Kjemtrup et al., 1995) can be secreted support this view. A possible target for βGLU1 is callose, which is a substrate for the enzyme (Stone and Clarke, 1992). Callose has been found in the impermeable covering layers of seeds of some dicotyledonous species, e.g. Cucumis melo (Welbaum et al., 1995; Yim and Bradford, 1998), but not in others, e.g. tomato and pepper (Beresniewicz et al., 1995). The presence of callose or other potential substrates of βGLU1 in the endosperm of tobacco has not been demonstrated and is an interesting area for further investigation.

The effect of after-ripening on endosperm could involve βGLU1

We found that after-ripening of Havana 425 tobacco seed had two effects on germination: it breaks photodormancy, which blocks germination prior to testa rupture (Leubner-Metzger et al., 1996; Mohapatra and Johnson, 1978), and it modulates coat-enhanced dormancy, which involves endosperm rupture. Little is known about the molecular basis for dormancy or the modulation of dormancy during after-ripening (Bewley, 1997a; Li and Foley, 1997). Although several genes that are differentially expressed in imibed dormant and non-dormant seeds have been identified, none have been shown to be directly involved in the maintenance or breaking of dormancy (Bewley, 1997a; Li and Foley, 1997). Over-expression of βGLU1 during germination replaced the after-ripening effect on endosperm rupture, but did not influence photodormancy. Endogenous production of ABA is needed to establish and maintain dormancy in many species including tobacco (Artsaenko et al., 1995; Bewley, 1997a; Fry et al., 1999; Grappin et al. 2000; Koornneef and Karssen, 1994; Li and Foley, 1997; Rock and Quatrano, 1995). The onset of dormancy in tobacco is correlated with a peak in ABA content, which declines rapidly during further seed maturation (approximately 15–25 DAP) (Jiang et al., 1996; Phillips et al., 1997; Yamaguchi-Shinozaki et al., 1990). The fresh tobacco seeds we used were sampled at approximately 40 DAP, which is after maturation and establishment of primary dormancy is complete. After-ripening is often correlated with a further decline in ABA content and decreased sensitivity to ABA (Benech-Arnold et al., 1999; Bewley, 1997a; Grappin et al. 2000; Hilhorst, 1995; Li and Foley, 1997; Ren and Kermode, 1999).

Recent work with Nicotiana plumbaginifolia suggests that de novo synthesis of ABA in imibed fresh seed also contributes to dormancy (Grappin et al. 2000). ABA treatment at concentrations in the range found in mature wild-type tobacco seeds transcriptionally down-regulates βGLU1 expression in the micropylar endosperm and markedly delays endosperm rupture (Leubner-Metzger et al., 1995). Moreover, expression of host βGLU1 in control seeds, which is down-regulated by ABA, was increased by after-ripening; whereas expression of the Cat1-regulated βGLU1 gene in sense seeds, which is up-regulated by ABA, was decreased by after-ripening (Table 1). This leads us to speculate that the expression of βGLU1 needed for endosperm weakening is inhibited by ABA present in the fresh control seed. During after-ripening, decreasing ABA levels and decreasing sensitivity to ABA eventually permit βGLU1 expression, which results in the release of dormancy (Figure 2).

Experimental procedures

Plasmid construction and plant transformation

The chimeric sense-βGLU1 construct KSG7 was obtained by transcriptional fusion between the 2.7 kb EcoRI–BamHI genomic DNA fragment of pCBI1 that contains the promoter of the castor
bean Cat1 gene (Suzuki et al., 1994; Suzuki et al., 1995) and the 2.9 kb BamHI–SphI genomic DNA fragment of the βGLU1B (Gib) gene which contains an introduced BamHI site located at the transcription start site, the entire coding sequence, the intron and 0.9 kb of 3’ flanking region (Hart et al., 1993). The resulting 5.6 kb EcoRI–SphI DNA fragment replaced the cauliflower mosaic virus (CaMV) 35S promoter in the pDH51 vector (Pietrzak et al., 1986) and is terminated by the CaMV 35S RNA polyadenylation signal. The binary vector pCIB200 (Neuhaus et al., 1992), which carries the chimeric neomycin phosphotransferase gene (NptII) under the control of the nopaline synthase (Nos) promoter and terminator, was linearized in the polylinker with KpnI and EcoRI and ligated to the KpnI–EcoRI fragment that contained the chimeric sense-βGLU1 gene. The resulting expression vector pKSG7 carried the chimeric sense-βGLU1 and Nos–NptII genes transcribed in the same direction.

The methods for introducing the pCIB200 expression vectors into Agrobacterium tumefaciens, Ti-plasmid transformation of Nicotiana tabacum L. cv. Havana 425 leaf discs, regeneration of plants, and segregation tests using the kanamycin resistance marker have been described previously (Beffa et al., 1993). TC1B1 transformants obtained with the empty-vector plasmid pCIB200 were used as controls. Segregation tests were performed with S1 seeds obtained by self-fertilization of independent primary transformants. Homozygous, monogenic S1 seeds were obtained by self-fertilization of S0 plants using kanamycin resistance as the marker.

Germination experiments

Seed from mature capsules of wild-type or transformed Havana 425 tobacco were used either at approximately 40 DAP (fresh seed) or after at least 6 months of dry storage at room temperature (after-ripened seed), as indicated. Germination analyses was performed as described by Leubner-Metzger et al. (1998). In brief, 100–150 seeds were sown on plastic Petri dishes containing filter paper wetted with a nutrient solution supplemented as indicated with 50 µg·ml⁻¹ kanamycin, 100 µg·ml⁻¹ Cliforafan (Hoechst-Pharma AG, Zürich, Switzerland), and 1 or 10 µM cis-(-):abscisic acid (ABA). Petri dishes were incubated at 25°C in continuous white light (3000 lux, Philips ‘TLD’ 35 W/33 lamps) or in darkness. After scoring for germination, seeds were stored at −80°C for subsequent analysis.

Analysis of proteins and RNA

Procedures for extracting proteins, assays for enzyme activity, immunoblot analysis, protein determination, preparation of total RNA and RNA-blot hybridization have been described previously (Leubner-Metzger et al., 1995). In brief, βGLU activity was assayed radiometrically using [1H]-laminarin as the substrate. The rabbit anti-tobacco βGLU antibody used for immunoblot analysis detects the class I, class II and class III isozymes of the enzyme (Beffa et al., 1993; Neuhaus et al., 1992). The ‘ECF Western blotting system’ (Amersham Pharmacia Biotech, Amersham, UK) was used for quantitative immunoblot analysis. The DNA probes used for RNA-blot hybridization were the 1 kb Psal fragment of the tobacco βGLU1 cDNA PGL43 (Shinshi et al., 1988) and the 1.8 kb EcoRI fragment of genomic DNA encoding tomato 18S ribosomal RNA (Schmidt-Puchta et al., 1989). Hybridized membranes were washed at high stringency. Signals were detected and quantified with a Phospholmager (Molecular Dynamics, Sunnyvale, CA, USA) and corrected for RNA loading based on the 18S ribosomal RNA signal.

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References


Role of $\beta$,1,3-glucanase in seed germination


